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Development of Radiolabeled Recombinant Erythropoietin for Receptor Studies

A.R. Jalilian[*1], H. Sadeghpour[2], M. Akhlaghi[3], E. Gholami[1]

1. Nuclear Science and Technology Research Institute, AEOI, P.O.Box: 14155-1339, Tehran-Iran
2. Department of Medicinal Chemistry, Faculty of Pharmacy, Shiraz University of Medical Sciences, P.O.Box: 158371345, Shiraz - Iran
3. Research Institute for Nuclear Medicine, Tehran University of Medical Sciences, P.O.Box: 1411713137, Tehran - Iran

Abstract: The study of human erythropoietin receptor is of great importance in evolution, carcinogenesis as well as endocrine research. In this study the production and quality control of human recombinant erythropoietin (EPO) has been reported. EPO was successively labeled with [^{67}Ga]-gallium chloride after conjugation with freshly prepared cyclic DTPA-dianhydride (ccDTPA). The best results of the conjugation were obtained by the addition of 0.5 ml of a EPO pharmaceutical solution (in phosphate buffer, pH=8) to a glass tube pre-coated with DTPA-dianhydride (0.01 mg) at 25°C with continuous mild stirring for 30 min followed by HPLC/RTLC control and biological biodistribution in normal rats. Under optimized conditions, radio-thin layer chromatography (RTLC), instant thin layer chromatography (ITLC) and high performance liquid chromatography (HPLC) showed overall radiochemical purity of higher than 96% (specific activity=300-500 MBq/mg, labeling efficiency 77%). Preliminary in vivo studies in normal rat specimen demonstrated a high liver, lung spleen, kidney as well as long bones uptake of the tracer; the fact which is consistent with the reported EPO receptor sites. This tracer can be used in nuclear medicine and biological studies for evaluation of EPO in vitro and/or in vivo.

***Keywords**: Radiogallium, EPO, Radiolabeling, Biodistribution, Radiotracer*

1. Introduction

Erythropoiesis is a highly efficient, multi-step process for forming red blood cells (RBC) from multipotent hematopoietic stem cells in which erythropoietin (EPO) plays a central role [1]. EPO is a 34 kDa glycoprotein whose amino acid sequence is highly conserved among mammals (91% identical in monkeys, ~80% identical in rats and mice) [2] (Fig. 1).

EPO is mainly produced by the kidney in response to hypoxia and the primary regulator of red blood cell production and is indispensable for terminal differentiation of erythroid progenitors. It controls proliferation, maturation and also survival of erythroid progenitor cells.

Absence of EPO or the EPO receptors (EpoR) results in interruption of definitive erythropoiesis in the fetal liver, [3] defective cardiac development, [4] and eventual death at about embryonic day 13.5 [5].

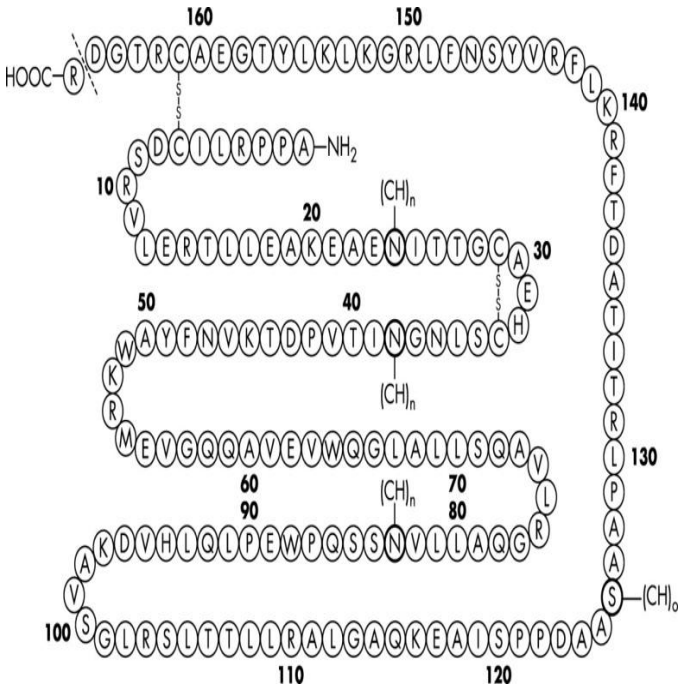

Fig. 1. Peptide sequence of erythropoietin [27].

*email: ajalili@aeoi.org.ir
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In animal specimens with a disrupted EPO system, the developing heart displays ventricular hypoplasia, defects in the interventricular septum and abnormalities of the vascular network [5]. EPO can cross the blood-brain barrier and can decrease brain or spinal cord damage in conditions such as, blunt trauma of the head [6] or of the spinal cord [7] or of an acute ischaemic injury of the brain, the spinal cord or retina [8], multiple sclerosis or status epilepticus [9] and it can prevent or partially reverse experimental diabetic neuropathy [10]. In vivo and in vitro analyses have shown that these neuroprotective effects are associated with a strong inhibition of apoptosis [7] and with neurotrophic activity of EPO [11].

The expression of EpoRs in various other organs such as endometrium, ovaries and other estrogen-dependent tissues, has been recently reported and raised the growth hormone-like effects of this molecule [12, 13]. The relative proportions and quantities of the erythropoietic and nonhematopoietic EpoRs in the body are not known nor may the manner in witch these quantities change during development. Few reports on the production and use of radiolabeled EPO are available. 125I-EPO has been prepared and used in study of GI absorbtion in neonates, revealing EpoRs presence in GI tract [14], pharmacokinetic studies in animal models [15], study of mitogenic/chemotactic EPO effects on endothelial cells [16] and EpoR binding on cell surface of some erythroleukemias [17].

Moreover, EPO and EPO-R were reported to be expressed in a variety of malignant human cell lines and solid tumors, including breast, prostate, ovarian, uterine and renal cancers [18, 19, 20, 21]. In order to obtain a radiolabeled conjugate for use in imaging/biodistribution studies of EpoR in normal and malignant models using metallic PET or SPECT radioisotopes, ^{67}Ga-labeled EPO was prepared.

The interesting physical properties and availability of gallium-67 make it an interesting nuclide for radiopharmaceutical research [22]. The increasing trend in the production and use of PET radionuclides in nuclear medicine has offered researchers further opportunities to focus on the production of new Ga-radiopharmaceuticals for feasibility studies using Ga-67 for their future PET gallium homologs.

In continuation of research program on the radiolabeling of peptides and hormones including EPO [23], a precise labeling strategy was employed using freshly-prepared DTPA cyclic dianhydride, with various EPO concentrations and utilizing available Gallium-67 for optimization of radiolabeling conditions due to longer half life in contrast to PET gallium radionuclides. Finally, an optimized radiolabeling method for developing a highly reactive DTPA-conjugated EPO for possible diagnostic studies was introduced.

2. Materials and Methods

2.1 Materials

^{67}Ga was obtained from Agricultural, Medical and Industrial Research School (AMIRS), 30MeV cyclotron (Cyclone-30, IBA). Sephadex G-50, sodium acetate, phosphate buffer components methanol and ammonium acetate were purchased from Sigma-Aldrich Chemical Co. (U.K.). Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. EPO was a pharmaceutical sample purchased from Pouyesh Darou Co. (Tehran, Iran) and was used without further purification. Radio-chromatography was performed by counting different 5mm slices of polymer-backed silica gel paper using a high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT equipped with two detector systems, flow scintillation analyzer (Packard-150TR) and UV-visible (Shimadzu) using Whatman Partisphere C-18 column 250×4.6mm, Whatman, NJ, USA. Calculations were based on the 184keV peak for ^{67}Ga. All values were expressed as mean±standard deviation (Mean±SD) and the data were compared using student T-test. Statistical significance was defined as $P<0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn.

3. Conjugation of Cyclic DTPA Di-Anhydride with Human Recombinant EPO

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated to the EPO using a small modification of the well-known cyclic anhydride method [24]. Conjugation was performed at a 1:1 molar ratio. In brief, 20µl of a 1 mg ml^{-1} suspension of DTPA anhydride in dry chloroform (Merck, Darmstadt, Germany) was pipetted under ultrasonication and transferred to a glass tube. Chloroform was evaporated under a gentle stream of nitrogen. Commercially available EPO (5mg, 0.5ml, pH 8) was subsequently added and gently mixed at room temperature for 60min. Conjugation mixture was then passed through a Sephadex G-50 column (2×15cm, 2g in 50ml of Milli-Q® water) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280nm or visible folin-phenol colorimetric assay. The fractions containing the highest concentration of the immunoconjugate were chosen for radiolabeling and kept at 4˚C.

4. Radiolabeling of EPO Conjugate with ^{67}Ga

The EPO conjugate was labeled using an optimization protocol according to the literature [25]. Typically, 37-40 MBq of ^{67}Ga-chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. To the Ga containing vial, conjugated fraction was added in 1ml of phosphate buffer (0.1 M, pH=8) and mixed gently for 30 seconds. The resulting solution was incubated at room temperature for 30minutes. Following incubation, the radiolabeled EPO conjugate was checked using ITLC/RTLC methods for the purity. In case of significant presence of impurities, the sample can be purified using gel filtration as described above. Control labeling experiments were also performed using $^{67}GaCl_3$, and DTPA with $^{67}GaCl_3$. Both reaction mixtures were passed through separate gel filtration columns and eluted with phosphate buffer solution (PBS). In case of gel filtration, the fraction showed the presence of protein used in the other experiments (n=3).

5. Quality Control of [^{67}Ga]-EPO

- Paper chromatography: A 5µl sample of the final fraction was spotted on a chromatography paper (Whatman No. 1. Whatman, Maidstone, UK), and developed in a mixture of 1mM DTPA in DDH_2O as the mobile phase.
- High performance liquid chromatography: HPLC was performed on the final preparation by means of acetate buffer solution (50mM pH. 5.5) as eluent (flow rate: 1ml/min pressure: 130 KgF/cm^2) for 20min in order to elute low molecular weight components. Radiolabeled peptide was eluted employing a gradient of the latter solution (100 to 0%) and citrate buffer solution (50mM, pH. 4, 0 to 100%) utilizing reverse stationary phase. Any remaining free Ga^{3+} cation could be complexed with citrate anion, however pre-complexed Ga-DTPA-EPO was not challenged with the addition of citrate chelate. At neutral pH the binding constant for gallium citrate was approximately log K=10.0 and for gallium DTPA it was approximately log K=25.7 [26].

6. Stability Testing of the Radiolabeled Compound

Stability of ^{67}Ga-DTPA-EPO in PBS was determined by storing the final solution at 4˚C for 14 days and performing ITLC analysis to determine radiochemical purity. Frequent ITLC analyses were made. Furthermore, the stability of the conjugated DTPA-EPO stored at –20˚C for more than 1 month was investigated. ITLC analysis of the conjugated product was carried out to monitor degradation products or other impurities. After subsequent ^{67}Ga-labelling of the stored conjugated product, both labeling efficiency and radiochemical purity were determined.

7. Stability Testing of the Radiolabeled Compound in the Presence of Human Serum

Labeled compound stability in freshly prepared human serum was assessed by gel filtration on a Sepharose column (1×30cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5mL/min at room temperature; 0.5mL fractions were collected.

8. Biodistribution of ^{67}Ga-DTPA-EPO in Normal Rats

To determine its biodistribution, ^{67}Ga-DTPA-EPO was administered to normal rats (NMRI) purchased from Razi Institute, Karaj, Iran. A volume (50µl) of final ^{67}Ga-DTPA-EPO solution containing 40±2µCi radioactivity was injected intravenously to rats through their tail vein (n=5). The animals were sacrificed at regular intervals (3, 22, 44 and 110 h), and the specific activity of different organs was calculated as a percentage of urea under the curve of 184keV peak per gram.

9. Results and Discussion

Conjugation of EPO with DTPA Cyclic Di-Anhydride and Radiolabeling of EPO with ^{67}Ga

The labeling yield of ^{67}Ga-DTPA-EPO has been studied in the wide range of EPO/DTPA ratios in order to optimize the process and to improve ^{67}Ga-DTPA-EPO performance in vitro. The overall radiolabeling efficiency was over 77% and the specific activity was kept in the range of 300-500 MBq/mg.

The conjugated ^{67}Ga-DTPA-EPO fractions containing the maximum protein content were mixed with ^{67}Ga-$GaCl_3$ solution, vortexed and kept at room temperature. Small fractions were taken from this mixture and tested by RTLC to find the best time scale for labeling. After an hour, free ^{67}Ga/conjugated ^{67}Ga ratio in the labeled sample remained unchanged. The mixture was then passed through another Sephadex G-50 gel filtration column in order to remove trace amounts of unbound ^{67}Ga cation.

The eluted fractions were checked by Folin-Colciteau® reagent and for the presence of radioactivity in order to determine the ^{67}Ga-DTPA-EPO containing fractions. Also by using folin method which consisted of the maximum radioactivity, the fraction with the maximum absorbance was chosen as the suitable final product with appropriate specific activity for animal tests. The radiolabeling reached 90% after 60min. Fig. 2. demonstrates the RTLC scheme of free Ga^{3+}, Ga-DTPA and radiolabeled protein.

At this stage the mixture was tested by HPLC in order to determine the radiochemical purity before administration to rodent models. Fig. 3. shows the HPLC chromatogram of ^{67}GaDTPA and the final solution. The fast eluting component (3.12min) was shown to be a mixture of free ^{67}Ga and ^{67}GaDTPA which were washed out on reverse stationary phase. Both compounds are ionic, so they are eluted at the same retention time. The radiolabeled protein was finally washed out within 12.77 minutes.

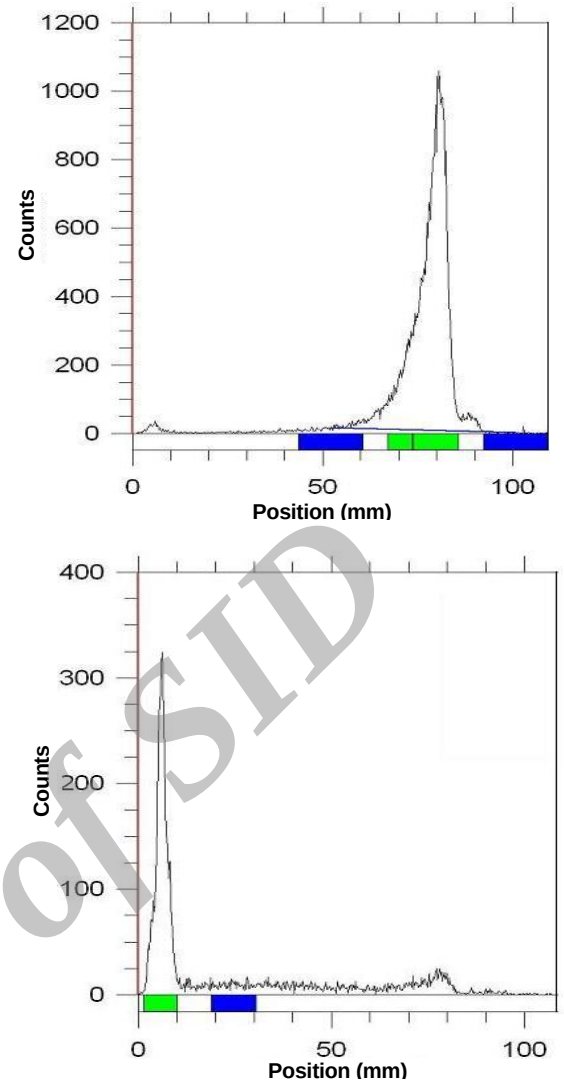

Fig. 2. RTLC radiochromatograms for free $^{67}Ga^{3+}$ and/or ^{67}GaDTPA (left) and ^{67}Ga-DTPA-rhuEpo (right) in 10mM DTPA solution under optimized conditions.

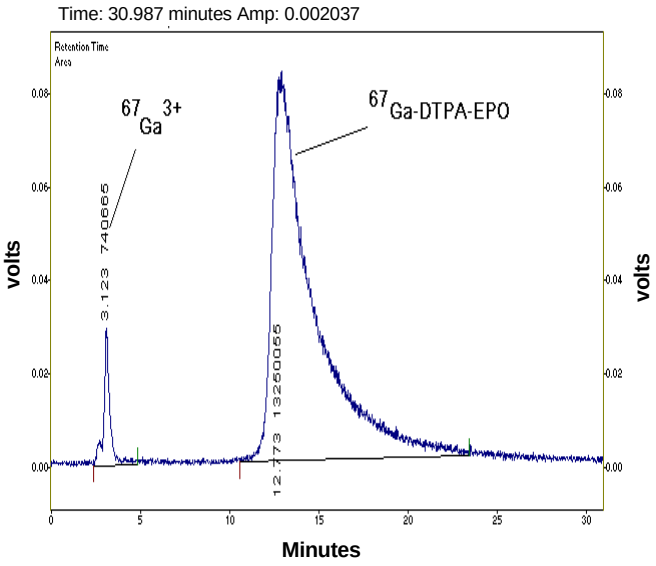

Fig. 3. HPLC radiochromatogram for ^{67}Ga-DTPA-rhuEpo in optimized conditions.

10. Stability of Radiolabeled Protein in Vitro

The stability of the radiolabeled protein in vitro was determined after challenge with phosphate-buffered saline and serum. ITLC analysis showed that the proteins retained the radiolabel over a period of several hours, indicating that the Ga-protein chelate was of high stability. These results were confirmed by gel filtration chromatography. After incubation of [^{67}Ga]-DTPA-EPO with PBS for 2h, almost all of the radioactivity was eluted in the same position as [^{67}Ga]-DTPA-EPO; there was no evidence for large-scale release of free Ga. Similarly, gel filtration chromatography of ^{67}Ga-DTPA-EPO after a 2h incubation with human serum showed that the radioactivity was still eluted in the same position. Thus, there was no evidence for either degradation or transchelation of ^{67}Ga to other serum proteins over a period consistent with the normal blood clearance time of EPO.

11. Biodistribution Studies

The distribution of [^{67}Ga]-DTPA-EPO among tissues were determined in normal rats. A volume (0.1ml) of [^{67}Ga]-DTPA-EPO solution containing 4.4-5.2 MBq radioactivity was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a dose calibrator with a fixed geometry. The animals were sacrificed by ether asphyxiation at selected times after injection.

Since the slight radioactivity of each organ due to its low amount could not be determined by regular dose-calibrators, for a more precise method, the activity was measured by an HPGe spectroscopic based on an apparent photopeak produced by Ga-67 decay (184keV for instance), and then considering the area under the curve for this photopeak, the branching ratio and finally the efficacy of the system would lead to the exact specific activity of the organs.

Two hours post injection, very low amount of activity is excreted via the kidneys that could be due to the presence of small amounts of Ga^{3+} GaDTPA and/or minor dissociation of the protein conjugate. The major organs of deposition are the liver, lung and spleen. The liver is a major destination of most of radiolabeled proteins, although the lungs and spleen also demonstrate the major uptake demonstrating a reticuluendothelial system uptake. The kindneys, however, have shown to possess EPO receptors among them as it has been reported previously (Fig. 4).

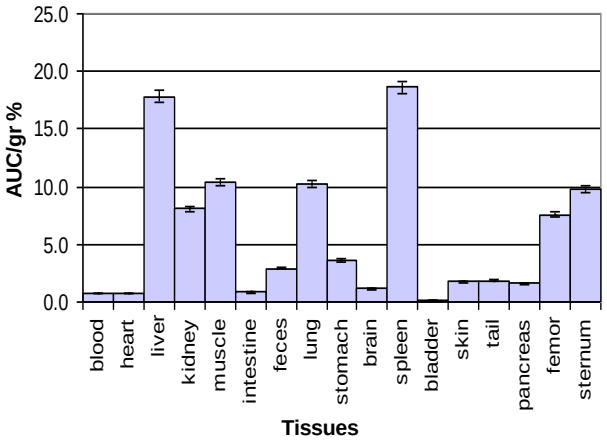

Fig. 4. Bio-distribution of ^{67}Ga-DTPA-rhuEpo in normal rats 2 h post-injection; AUC: area under curve of the 184keV peak in gamma spectrum.

The other interesting organ of accumulation seems to be the bone, both femur and sternum show significant uptake, which contain bone marrow, considered as the major targeting organ for EPO hormone.

12. Conclusions

EPO was successively labeled with [^{67}Ga]-gallium chloride after conjugation with freshly prepared cyclic DTPA-dianhydride (ccDTPA). Total labeling and formulation of [^{67}Ga]-DTPA-EPO took about 60minutes. The best results of the conjugation were obtained by the addition of 0.5ml of a EPO pharmaceutical solution (in phosphate buffer, pH=8) to a glass tube pre-coated with DTPA-dianhydride (0.01mg) at 25˚C with continuous mild stirring for 30min A radiochemical purity of higher than 96% was detected by HPLC. In 2-3 hours, radiolabeled hormone was cleared from the blood circle. The major organs of deposition were the liver, lung and spleen. The liver was a major destination of most of radiolabeled proteins, although the lungs and spleen also demonstrated the major uptake indicating a reticuluendothelial system uptake. The kidneys however had shown to possess EPO receptors among them as it had been reported previously. The other interesting organ of accumulation seems to be the bone, both femur and sternum show significant uptake, which contained bone marrow, considered as the major targeting organ for EPO hormone.

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